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DAMPs and autophagy

Cellular adaptation to injury and unscheduled cell death

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Abbreviations: ATG, autophagy-related; DAMP, damage-associated molecular pattern molecule; PAMP, pathogen-associated molecular pattern; ROS, reactive oxygen species

Autophagy is a lysosome-mediated catabolic process involving the degradation of intracellular contents (e.g., proteins and organelles) as well as invading microbes (e.g., parasites, bacteria and viruses). Multiple forms of cellular stress can stimulate this pathway, including nutritional imbalances, oxygen deprivation, immunological response, genetic defects, chromosomal anomalies and cytotoxic stress. Damage-associated molecular pattern molecules (DAMPs) are released by stressed cells undergoing autophagy or injury, and act as endogenous danger signals to regulate the subsequent inflammatory and immune response. A complex relationship exists between DAMPs and autophagy in cellular adaption to injury and unscheduled cell death. Since both autophagy and DAMPs are important for pathogenesis of human disease, it is crucial to understand how they interplay to sustain homeostasis in stressful or dangerous environments.

Introduction

A fundamental challenge in studies of human health and its derangement or pathology is elucidating the molecular, bioenergetic and anatomical basis of homeostatic imbalance. Homeostasis, a term first introduced by Claude Bernard in the 1860s, is a biological system's ability to maintain a relatively stable internal milieu in a fluctuating external environment.[1] Cells, as the structural and functional units of tissues and organs, must maintain homeostasis in response to various physiological and pathological stressors. How a cell maintains homeostasis in the face of stress or damage is briefly reviewed here. We provide a brief overview of the process and function of adaptation to such stressors and focus on the complex relationship between autophagy and DAMPs in the cellular response to injury and death (**Fig. 1**).

Cellular Adaptation, Injury and Cell Death

To maintain a stable intracellular environment, cells can respond to physiological stressors or pathological stimuli in various ways, ranging from the activation of survival pathways to the initiation of cell death.[2,3] This response causes changes in the number, size, morphology and types of cells within tissues to maintain viability and function. These processes, collectively called cellular adaptation, include atrophy (decrease in cell size), hypertrophy (increase in cell size), hyperplasia (increase in cell number), and metaplasia (change in cell type). Physiological adaptation includes responses of cells to stimulation by hormones or endogenous chemical mediators, whereas pathological adaptation usually represents a response to stress that allows cells to modulate their structure and function and thus limit damage and initiate repair. Reversible injury is usually mild, and following removal of the adverse influence, the cell reverts to its normal steady-state. If it cannot recover, the cell either enters a period of senescence or progresses to cell death.

Many types of cell death are described, including necrosis, necroptosis, pyroptosis, entosis and apoptosis,[4,5] as well as the recently identified ferroptosis, an iron-dependent form of non-apoptotic cell death.[6] Cell death can be classified according to its morphological appearance, enzymological criteria and functional aspects, or its immunological characteristics.[4,5] Although several common biochemical events are observed in the process of cell death, the mechanisms leading to cell death caused by individual stressors may vary. Of note, many proteins currently assumed to be cell death proteins have distinctly different functions, including prosurvival roles.[7] For example, CASP8, the initiator caspase of the death receptor pathway of apoptosis, has significant non-apoptotic roles in embryonic development and prosurvival functions.[8-10] Deletion of the apoptotic gene can, in some instances, promote tumorigenesis.[11] In addition, apoptotic cells not only suppress, but also promote an inflammatory response, which is dependent on the context of the tissue microenvironment in which cell death occurs.[12,13]

Autophagy: A Multifaceted Lysosomal Degradation System for Bulk and Selective Recycling

The two major intracellular degradation systems are the ubiquitin-proteasome system and autophagy. The ubiquitin-proteasome system is used for rapid degradation of proteins, whereas autophagy is primarily responsible for the lysosomal degradation of long-lived proteins and damaged organelles. The term

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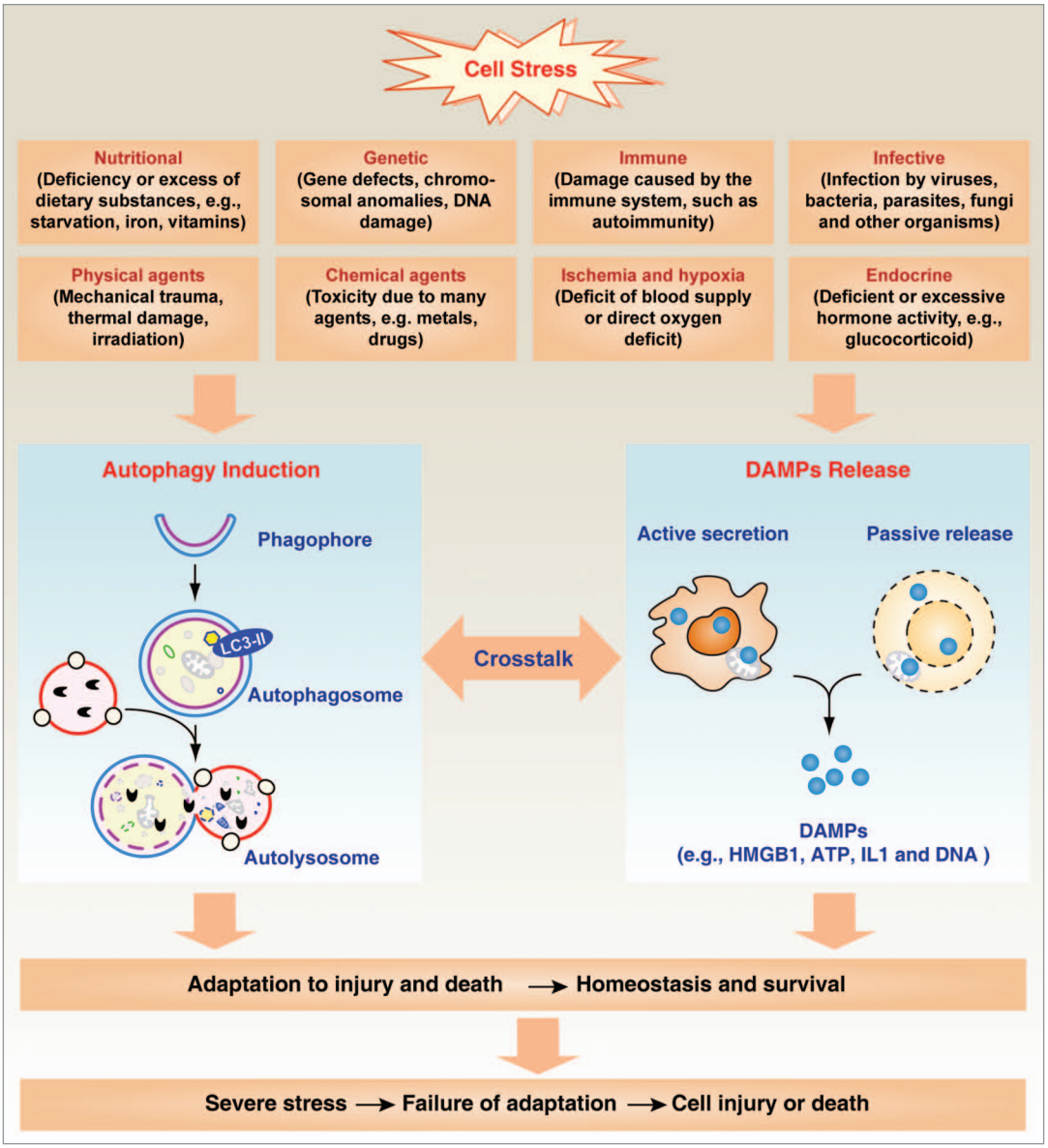

Figure 1. Cellular response to stress and injurious stimuli. When cells are faced with physiological or pathological stresses, they can undergo adaptation, achieving a new steady-state and preserving viability and function. Induction of autophagy and DAMPs release are stress responses to injury. If the adaptive capability is exceeded or if the external stress is inherently harmful, cell injury or death develops.

"autophagy" is derived from the Greek words "phagy" meaning eat and "auto" meaning self. Christian de Duve, a Nobel Prize-winning cytologist and biochemist, first coined the term "autophagy" in 1963 following electron microscopy morphological observations.[14] Autophagy is an evolutionarily conserved, multistep, degradative process conserved among yeast, plant and animal cells. In mammalian cells, types of autophagy include macroautophagy (hereafter referred to as autophagy), chaperone-mediated autophagy and microautophagy, which are frequently interconnected and share several common components. In general, autophagy is the cellular catabolic process that leads to the removal of damaged biological macromolecules and organelles through engulfment and fusion with lysosomes. The autophagic structures, including phagophores, autophagosomes and autolysosomes, have distinct morphologies and their formation is primarily controlled by members of the autophagy-related (Atg) proteins,[15] most of which were initially identified in yeast. The origin of the autophagosomal membrane includes the plasma membrane, the endoplasmic reticulum, mitochondria and the Golgi apparatus.[16]

Autophagy is not only a nonselective bulk degradative pathway, but also a selective pathway. Selective autophagy such as mitophagy (degradation of mitochondria)[17] and xenophagy (degradation of intracellular pathogens)[18] is mediated by specific autophagic adaptor proteins (e.g., sequestosome 1 [SQSTM1/p62], BCL2/adenovirus E1B interacting protein 3-like [BNIP3L/NIX], calcium binding and coiled-coil domain 2 [CALCOCO2/ NDP52], neighbor of BRCA1 gene 1 [NBR1], optineurin [OPTN], and

lectin, galactoside-binding, solulble, 8 [LGALS8/galectin 8]).[19] Autophagy is a housekeeping survival mechanism that promotes programmed cell survival,[20] with a protective function in the setting of stress to sustain homeostasis by maintaining cellular integrity and promoting efficient cellular function, distinct from apoptosis or programmed cell death. When stress severity or duration increases, however, it may promote cell death. The term "autophagic cell death" was first established based on observations of increased autophagic markers in dying cells.[4,5] In many cases, it is agreed that this "autophagic cell death" is cell death *with* autophagy rather than cell death *by* autophagy.[21,22] Autophagy is involved in the regulation of diverse biological processes, including development, differentiation, cell cycle progression, cell death, immunity, inflammation and metabolism, and is dysfunctional in several diseases.[23-30] Thus, the accurate and facile identification and quantification of autophagy is necessary to make progress in this rapidly developing field.[31,32]

DAMPs: Endogenous Molecules Released by Stressed Cells

The immune system is composed of a network of cells, tissues and organs that work together to recognize tissue stress, damage and pathogens often portrayed as "non-self/stranger" or "damaged self/danger" signals. Charles Janeway, Jr. predicted the first exogenous signal in 1989, which we now term collectively "pathogen-associated molecular pattern" (PAMP) molecules.[33] Polly Matzinger predicted the latter endogenous form in 1994, which we now term "damage-associated molecular pattern" (DAMP)[34,35] molecules. Most DAMPs are nuclear or cytosolic proteins, including the chromatin-associated high mobility group box 1 (HMGB1), the S100 family of calcium-binding proteins, heat shock proteins, interleukin 1 (IL1) family members (e.g., IL1A and IL33), and histones.[36] Examples of nonprotein DAMPs include adenosine triphosphate (ATP), DNA, RNA, uric acid, hyaluronan and heparin sulfate.[36] Increasing evidence suggests that mitochondria are an important source of DAMPs (e.g., mitochondrial DNA and transcription factor A, mitochondrial/TFAM, a structural and functional homolog of HMGB1).[37] These DAMPs have well-defined functions with several common characteristics: (1) they are passively leaked from injured or dying cells or the surrounding tissue matrix; (2) they can be actively secreted by immune cells through various nonclassical pathways; (3) their biological activities are mediated by interaction with polygamous pattern recognition receptors including toll-like receptors and the advanced glycosylation end product-specific receptor (AGER/RAGE), or post-translational modification such as redox modulation; (4) they serve as so-called "signal 0's" to promote immune and stress responses to restore homeostasis; and (5) excessive levels of DAMPs may cause cell injury, limit normal organ function (systemic autophagic syndromes), and thereby promote organismal death, paradoxically in a setting of heightened, but excessive, programmed cell survival. Increased plasma levels of DAMPs are associated with several inflammatory-related diseases such as rheumatoid arthritis, systemic sepsis, atherosclerosis, hepatitis, diabetes and cancer.[38,39] It is thus critical to explore the mechanisms of DAMP release and assess their beneficial or maladaptive biological roles in the setting of disease.

Autophagy-Mediated DAMP Release and Degradation

The release of DAMPs as soluble messengers is a fundamental mechanism for cell-to-cell communication and regulation within the immune system during stress. Most cellular DAMPs are released by a nonclassical or unconventional secretory route in which they are released through the plasma membrane without passing through the traditional secretory protein route, including the Golgi complex.[40,41] Recently, multiple nonconventional transport pathways have been invoked, including secretory lysosomes, endosomes, membrane blebbing, multivesicular body-derived exosomes, ABC transporters and autophagy[42-44] as vehicles for exteriorizing following cell stress without cell death, necessarily. Increasing evidence indicates that autophagy regulates release and degradation of DAMPs including HMGB1, ATP, IL1B, and DNA in several cell types (**Fig. 2**).

HMGB1. HMGB1, an abundant chromatin-binding protein, is the best-characterized DAMP.[36,45] It can be released from dying cells (e.g., necrosis and late-stages of apoptosis) and activated immune cells [macrophages, neutrophils, eosinophils, natural killer cells, dendritic cells (DCs), and platelets], and mediates the response to infection, injury, and inflammation. HMGB1 release is regulated by post-translational modification (e.g., acetylation, phosphorylation, ADP ribosylation and redox modulation) and activation of signal transduction pathways (e.g., mitogen-activated protein kinase and double-stranded RNA-activated protein kinase), and vesicle-mediated lysosome exocytosis. We and others have demonstrated that autophagy regulates the release and secretion of HMGB1 in several cell types in response to starvation, cytotoxic drugs, and PAMPs (**Fig. 2A**).[46-49] Reactive oxygen species (ROS)-dependent signals are required for autophagy-mediated HMGB1 release. Inhibition of autophagy by inhibitors (e.g., 3-methyladenine, bafilomycin A_1 and chloroquine) or genetic deletion/depletion of autophagy regulators (e.g., ATG5 and BECN1) inhibits HMGB1 release in fibroblasts, macrophages and cancer cells. Moreover, autophagy regulates monosodium urate crystal-induced HMGB1 release by neutrophil extracellular traps (**Fig. 2B**).[50,51] neutrophil extracellular traps are extracellular chromatin structures that entrap microbes and are composed of nuclear and granule constituents of neutrophils. Tanshinone IIA sodium sulfonate, originally derived from a well-known Chinese medicine used for treating cardiovascular disorders, induces clathrin- or caveolin-dependent endocytosis of exogenous HMGB1 and subsequently, autophagy-dependent degradation in macrophages (**Fig. 2C**).[52] In addition, epigallocatechin gallate, a major ingredient in green tea, inhibits HMGB1 release in macrophages by stimulating its autophagic degradation.[53] Collectively, these studies suggest that autophagy may play a central role in regulation of the cellular traffic, secretion and degradation of HMGB1.

ATP. ATP, the bioenergetic primary substrate or currency of metabolism, plays a central role intracellularly. In addition, extracellular ATP regulates many biological processes including cardiac function, neurotransmission, muscle contraction, immune

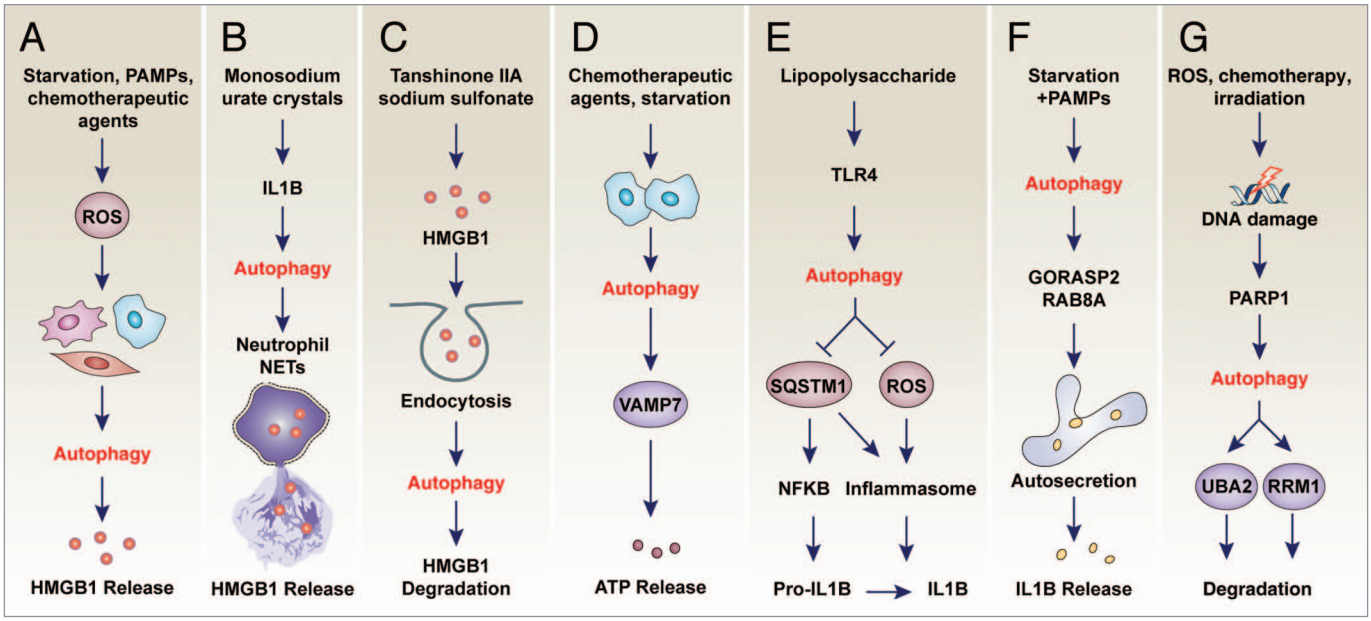

Figure 2. Autophagy-mediated DAMP release and degradation. (**A**) Autophagy regulates HMGB1 release in a ROS-dependent manner in fibroblasts, macrophages and cancer cells. (**B**) Autophagy regulates neutrophil extracellular trap-mediated HMGB1 release in neutrophils. (**C**) Tanshinone IIA sodium sulfonate facilitates endocytosis of exogenous HMGB1 and triggers HMGB1 degradation by autophagy in macrophages. (**D**) Autophagy is required for the release of ATP by cancer cells. (**E**) Autophagy as a mechanism to prevent exaggerated endotoxin-induced IL1B production in macrophages. (**F**) Autophagy promotes IL1B release through cell membrane targeted vesicular exocytosis. (**G**) Autophagy regulates DNA damage response by degradation of UBA2 and RRM1.

responses and inflammation.[54] In immune cells, ATP can trigger the activation of NLR family, pyrin domain containing 3 (NLRP3) and initiate the creation of an inflammasome and subsequent IL1B release in response to PAMPs (e.g., lipopolysaccharides and peptidoglycan). Dying autophagic cells have the ability to release ATP through the purinergic receptor P2X, ligand-gated ion channel, 7 (P2RX7) and K^+ efflux.[55] During recognition and engulfment by macrophages, ATP release from dying autophagic cells can induce an acute inflammatory response through the NLRP3 inflammasome activation and IL1B release.[55] It is not known, however, whether synergistic effects exist between ATP and other DAMPs such as HMGB1 on inflammasome activation. In cancer cells, autophagy is required for mitoxantrone and oxaliplatin-induced release of ATP from early transplantable tumors, which stimulates antitumor immune responses by recruiting DCs and lymphocytes (**Fig. 2D**).[56] In contrast, loss of ATG5 or ATG7 inhibits the release of ATP by tumor cells in response to chemotherapy, and prevents antitumor immune responses.[56] These findings contribute to our understanding of how cancer cells can be immunogenic in the hosts in which they arose.

Vesicular exocytosis contributes to ATP release, although the exact mechanism is unknown. A recent study indicates that vesicle-associated membrane protein 7 (VAMP7) is required for starvation-induced ATP release by the delivery and fusion of ATP-containing amphisomes with the cell membrane in cancer cells (**Fig. 2D**).[57] VAMP7-positive vacuoles colocalize with autophagosomes at the focal adhesion sites upon starvation, and microtubule-mediated trafficking participates in the transport of these VAMP7-labeled autophagic vesicles.[57] VAMP7 is a transmembrane protein that is a member of the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) family. Of note, SNARE proteins, which are essential for membrane fusion, are also required for formation and maturation of autophagosomes[58,59] as well as autophagosome-endosome/lysosome fusion,[60] suggesting that the core molecular machinery of autophagy shares components with other trafficking pathways.

IL1B. IL1B is usually considered as a mediator downstream of DAMPs although it has the primary characteristics of DAMPs. IL1B can be actively secreted by immune cells in response to exogenous and endogenous inflammatory stimuli or passively released by necrotic cells, and mediates innate and adaptive inflammatory responses to microbial invasion and tissue injury. The NLRP3 inflammasome is a cytosolic protein complex regulating the activation of CASP1, which in turn cleaves pro-IL1B into its active form and promotes IL1B release. ATG16L1 is essential for autophagy and is implicated in the susceptibility to inflammatory bowel disease, being mutated in many patients with Crohn disease of the bowel. ATG16L1 suppresses IL1B release in response to lipopolysaccharides (LPS) in macrophages,[61] suggesting an important role of autophagy in regulating IL1B release. The mechanism for this is thought to be involved in regulating pro-IL1B for lysosomal degradation, ROS production, activation of the NLRP3 inflammasome and SQSTM1 stability (**Fig. 2E**).[61-63]

LPS treatment of macrophages induces the recruitment of IL1B to autophagosomes, and the sequestered pro-IL1B is degraded when autophagy is activated by treatment with rapamycin.[62] Moreover, the autophagic adaptor protein SQSTM1

directly delivers ubiquitinated inflammasomes to complete their degradation in autophagosomes.[64] In contrast, inhibition of autophagy by 3-methyladenine (3-MA) induces activation of the NLRP3 inflammasome, and knockout of NLRP3 in bone marrow-derived DCs decreases IL1B release in response to LPS and 3-MA.[62] ROS are essential secondary messengers in induction of NLRP3 inflammasome activation. Autophagy is an important regulator of intracellular ROS generation by controlling mitochondrial quality.[65] ATG16L1-deficient macrophages generate higher levels of ROS and IL1B release in response to LPS,[61] and ROS scavengers prevent 3-MA-induced inflammasome activation.[62] In addition to its role in autophagy, SQSTM1 acts as a positive regulator of IL1B release by inducing nuclear factor of kappa light polypeptide gene enhancer in B-cells (NFKB) activation. A recent study indicates that ATG16L1 suppresses IL1B release by promoting degradation of SQSTM1 via CUL3/cullin 3-mediated proteasomal and autolysosomal degradation.[63] In aggregate, these studies suggest that autophagy suppresses IL1B release at multiple levels. Interestingly, the autophagy-based unconventional secretory pathway, namely autosecretion, is required for PAMP-induced IL1B release in the setting of starvation (**Fig. 2F**).[48] Loss of ATG5 inhibits starvation-induced IL1B release, thus requiring Golgi reassembly stacking protein (GRASP) paralogs GORASP2 or RAB8A.[48] Thus, autophagy plays a dual role in regulation of IL1B release in a context-dependent fashion. In addition, autophagy regulates release of IL1A and IL18 in several cell types, although the underlying mechanism remains to be clarified.[66]

Microtubule-associated protein 1 light chain 3 (LC3)-associated phagocytosis (LAP) is a newly discovered autophagic-phagocytosis "hybrid" process that utilizes a single-membrane structure.[19,67] LAP plays a role in the regulation of the immune response and promotes degradation of phagocytosed cellular corpses.[67,68] Macrophages lacking LAP due to inhibition of individual elements of the classical autophagy pathway (e.g., BECN1, ATG5, and ATG7) increase IL1B production following engulfment.[68] It remains unknown, however, whether the effect of inhibiting individual autophagy genes is due to a failure in conventional autophagy rather than in LAP per se.

DNA. Leakage of nuclear and mitochondrial DNA into the bloodstream during cell damage can activate the immune system and result in a variety of diseases including multiorgan dysfunction and failure in response to sepsis or trauma, neurodegenerative diseases, diabetes, cancer and aging.[69-71] DNA damage induces poly (ADP-ribose) polymerase 1 (PARP1)-dependent autophagy in the setting of oxidative stress, chemotherapy and irradiation.[72-74] Autophagy regulates the DNA damage response at multiple levels. Ubiquitin-like modifier activating enzyme 2 (UBA2/SAE2, a protein involved in repairing meiotic and mitotic double-strand breaks in DNA)[75] and ribonucleotide reductase M1 (RRM1/RNR1, an enzyme that catalyzes the formation of deoxyribonucleotides needed for DNA replication and repair)[76] can be directly degraded via autophagy (**Fig. 2G**). Moreover, deficiency of autophagic genes, including *ATG7*[77] and *BECN1*,[78] and UV radiation resistance associated gene (*UVRAG*),[79] increases DNA damage, genome instability and tumorigenesis. In addition, removal of micronuclei by autophagy, namely nucleophagy, may contribute to genomic stability.[80,81] Mitophagy, the specific autophagic elimination of mitochondria, has been implicated in the control of mitochondrial number and quality[17] and is thought to be critically important to prompt subsequent mitochondrial biogenesis. Mitochondrial DNA that escapes autophagic clearance can cause systemic and cardiac inflammation as well as heart failure.[82] Depletion of autophagic proteins (LC3B and BECN1) promotes the accumulation of dysfunctional mitochondria, mitochondrial DNA release and NLRP3 inflammasome activation in macrophages.[83] Thus, endogenous (genomic or mitochondrial) DNA that escapes degradation following enhanced autophagy results in innate immune activation.

Autophagy Regulation by DAMPs

Heightened autophagy is rapidly and uniformly initiated following various physiological and pathological stimuli. The mechanism and regulation of autophagy is extremely complicated and involves multiple signaling inputs. Recent studies suggest that DAMPs, including HMGB1 and ATP, are powerful autophagic stimuli and regulators. Exogenous HMGB1 and ATP promote autophagy in cancer and immune cells.[52,84-86] ATP induces P2RX7-dependent autophagy in monocytes/macrophages and microglial cells, which contributes to the elimination of intracellular mycobacteria[85] and release of autophagolysosomes/phagolysosomes into the extracellular space.[86] In addition, the autophagy inhibitor 3-MA has an inhibitory effect on the ATP-dependent release of IL1B in peripheral blood mononuclear cells,[87] suggesting that autophagy regulates ATP-induced inflammasome activation and cytokine production.

The mechanism of HMGB1-mediated autophagy has been well studied. The activity of HMGB1 in immunity, inflammation and autophagy depends on its redox status and expression of several cognate receptors.[88] Reduced HMGB1 protein promotes autophagy in an AGER/RAGE-dependent fashion, whereas oxidized HMGB1 promotes apoptosis with activation of CASP3.[54,55] Cytoplasmic HMGB1 is a BECN1-binding protein, which sustains BECN1-PtdIns3K complex activation during autophagy induction.[46] Tumor protein p53 (TP53) and unc-51-like kinase 1 (ULK1) have opposing roles in regulation of HMGB1-BECN1 complex formation in cancer cells.[89,90] Nuclear HMGB1 regulates expression of heat shock 27kDa protein 1 (HSPB1/HSP27).[91] As a cytoskeleton regulator, HSPB1 is critical for dynamic intracellular trafficking during autophagy and mitophagy. Loss of either HMGB1 or HSPB1 produces phenotypes similar to those characterized by mitochondrial fragmentation with decreased aerobic respiration and ATP production.[91] Both intracellular and extracellular HMGB1-mediated autophagy promote chemoresistance in several cancer cell types, including colon cancer, pancreatic cancer and leukemia.[89,90,92]

Concluding Remarks and Perspective

Cells have evolved several critically important strategies, such as induction of heightened autophagy promoted by DAMP release,

as defense mechanisms to respond to stressful conditions and sustain survival in hostile environments. The crosstalk between autophagy and DAMP release, in the setting of cell stress has been characterized. The autophagic machinery regulates DAMP release and degradation, contributing to the inflammatory response and subsequent immunity. Interestingly, DAMPs can also induce autophagy, contributing to chemoresistance and enhanced viral and bacterial removal. In general, autophagy is used to engulf nonspecific components, but it can also selectively degrade damaged organelles or invasive pathogens. Autophagy is regarded as a carefully regulated ATG-dependent process, characterized by specific morphological and biochemical features in which LC3 turnover plays a central role. Although many of the key autophagic proteins that are activated or inactivated in the autophagic pathways have been identified, the molecular mechanisms of action or activation/modification of these proteins are not fully understood and are the focus of continued research. Understanding the mechanisms of DAMPs and autophagy at the molecular level will provide deeper insights into various disease processes and suggest the development of novel therapeutic strategies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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